DETECTION OF IMMUNOMAGNETIC BEAD CAPTURED *ESCHERICHIA COLI* O157:H7 BY LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR[1]

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ABSTRACT

A modified approach using a light addressable potentiometric sensor (LAPS) method to detect Escherichia coli *O157:H7 in solutions is described. The bacterial cells were captured by the specific antibody coated on magnetic beads. The immuno paramagnetic beads (IMB) were then concentrated by a magnetic separator. Fluorescein-labeled anti* E. coli *antibody and urease-conjugated anti-fluorescein antibody were then used to label the cells captured by the beads. After labeling, the mixtures were rapidly filtered through 0.45 or 1.0 μm nitrocellulose or polycarbonate filters. The production of NH_3 from urea by the conjugated urease associated with the captured bacteria was then analyzed. The results indicate that IMB approach can be used to increase the specificity of the LAPS method for pathogenic bacteria detection. With this procedure, the presence of about one* E. coli *O157:H7 CFU per g of hamburger meat can be detected after a 6 h incubation.*

INTRODUCTION

Pathogenic microorganism contamination of foods is a serious public health concern in the United States. One heavily cited pathogen is the bacterium *Escherichia coli* O157:H7 for its ability to cause hemolytic uremic syndrome that may lead to kidney damage and failure and death (Fed. Regis. 1995). Thus, methods to rapidly and sensitively detect the presence of the *E. coli* and other pathogenic bacteria are needed. Numerous approaches for this need have been

[1] Mention of brand or firm names does not constitute endorsement by the U.S. Department of Agricultural over others of a similar nature not mentioned.

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devised (Feng 1992, 1996), and new approaches continued to be introduced. In general, the approaches involve the use of specific antibodies to capture the targeted microbes. Sensitive detection of immuno-captured microbes may be achieved through amplification of signals generated by either attached external labels or specific internal cellular contents of the cells. The former approach includes ELISA and many of its variations (Tsai *et al.* 1994; Wyatt *et al.* 1993). Cellular nucleic acid staining (Jacobsen *et al.* 1997) and ATP measurement are examples for the latter approach (Ward *et al.* 1986; Siragusa *et al.* 1996).

One of the former approaches involves the use of a light-addressable potentiometric sensor (LAPS) to detect intact viruses (Lee *et al.* 1993), bacterial spores (Dill *et al.* 1997), and bacteria (Libby and Wada 1989). LAPS technology has been incorporated with urease-linked immuno assay and Millipore filtration approaches to form the commercially available Threshold System (Molecular Devices, Sunnyvale, CA). Recently, we reported an application of using the Threshold System to detect *E. coli* O157:H7 (Gehring *et al.* 1998). The method involved the formation of a complex with the bacteria, fluoresceinated anti-*E. coli* O157 antibody, urease labeled anti-fluorescein antibody, and biotinylated anti-*E. coli* O157 antibody. Streptavidin was then used to link the complex to biotinylated bovine serum albumin on nitrocellulose membranes. The membrane assembly (sampling sticks) was then placed in urea containing solutions and the time course of the production of NH_3 shown as pH changes near the surface of the sensor was recorded as voltage changes. Light from a 10 KHz light emitting diode charges the silicon in the detector. The magnitude of the current generated depends on the potential across the sensor which may be changed by the pH increase due to the production of NH_3 resulting from the urease reaction. The method was able to detect about 10^4 to 10^5 live cells per mL of Tris-buffered saline (Gehring *et al.* 1998). Practical applications of this process in complex food systems have yet to be tested. A lack of rapid and effective methods to concentrate the antibody-bacterium complexes formed from a series of immuno reactions in food suspensions, may be the barrier for adopting the method for pathogenic bacteria detection.

In current work, we report a different approach to the above complex Threshold System procedure for detecting *E. coli* O157:H7. We used paramagnetic beads coated with antibodies specific to the bacteria to directly capture the bacteria in hamburger suspensions (Dynal Corp., Oslo, Norway). The captured bacteria were then further treated with fluorescein labeled anti-*E. coli* O157:H7 antibody and urease-labeled anti-fluorescein antibody. The beads with captured and labeled bacteria were rapidly concentrated either on 0.45 μm nitrocellulose or 1.0 μm polycarbonate filter membranes. Captured bacteria were then detected with the LAPS of a Threshold System. The method could detect the presence of about one CFU of *E. coli* O157:H7 per gram of hamburger after a six hour enrichment.

MATERIALS AND METHODS

Bacterial Growth and Enumeration

A loop of *Escherichia coli* O157:H7 (strain B1409, Center for Disease Control, Atlanta, GA) was collected from a slant and inoculated into 25 mL of brain-heart infusion (BHI) broth (Difco Inc, Detroit, MI) and incubated at 37C for 18 h with 160 rpm shaking. Cells were immediately placed on ice to halt growth. Bacterial samples were diluted 1:100 and 6 μL were placed on a Petroff-Hauser bacteria counting slide (Hauser Scientific, Horsham, PA). A center square on the slide of 0.2 $\times$ 0.2 mm was divided into 16 squares. Bacteria were counted in a random sampling of 5 of these squares. The averages of these counts were used to determine the total cell concentrations. Cells were diluted in sterile TBS and plated either on BHI media with agar or plates restrictive for *E. coli* O157:H7 (Fluorocult *E. coli* O157:H7 Agar, EM Science, Gibbstown, NJ).

Threshold System Detection

The operation principle and the basic construction of the Threshold system has been described in our previous report (Gehring 1998). To use this instrument for bacterial detection, the cells were first captured by use of IMB. To 1 mL bacterial samples in 1.5 mL eppendorf tubes, 15 μL of anti-*E. coli* O157 IMB (6×10^7 beads/mL) were added (Dynal Inc, Oslo, Norway). These tubes were gently shaken for 30 min and then placed in a magnetic particle concentrator (Dynal Inc). The concentrator was placed for 3 min on a rocker to concentrate the beads. Supernatant was removed and the bacteria-IMB pellets were stored over ice. Lyophilized urease labeled anti-fluorescein antibody was reconstituted in diluted special "assay buffer" that was supplied by the manufacturer Threshold System (Molecular Devices, Sunnyvale, CA). To 1 mL of this reconstituted solution, 10 μL (50 μg/mL) of fluorescein labeled goat anti-*E. coli* antibody (KPL, Gaithersburg, MD) was added. This solution was vortexed diluted to 10 mL with diluted "assay buffer". To each of the bacteria-IMB pellets in the centrifuge tubes, 1 mL of this final antibody cocktail solution was added. Tubes were then gently mixed for 15 min and beads reconcentrated by the magnetic particle concentrator on a rocker for 3 min. The supernatant was removed and the resulting bacteria-IMB pellets were resuspended in 1 mL of diluted "wash buffer" (a solution provided by Molecular Devices), and reconcentrated. These pellets were resuspended in 1 mL of the wash buffer. Up to 32 samples could be simultaneously concentrated using the 4 block, 8 well filter assembly of the Threshold Device (Molecular Devices, Sunnyvale, CA). Aliquots of 450 μL each of the final suspension were loaded into the wells and the vacuum manifold was turned on at the "low" setting for

approximately 5 min. The vacuum then was switched off and 450 μL of the "wash buffer" was added to each sample well. Additional 5 min of vacuum on "low" setting was applied to wash the packed complex. The samples were placed into the reader chamber which was filled with the "wash buffer" containing 100 mM urea. The time courses of pH change induced by the production of NH_3 near the surface of the bacteria-IMB complex sites were recorded for 120 s (factory set). The initial linear slopes of the time courses expressed as the rates of potential changes ($\mu V\ s^{-1}$) were used by the software to automatically calculate the LAPS values.

Detection of the *E. coli* on Beef Hamburger

To 225 mL of modified EC media (Difco Labs, Detroit MI), 25g of raw beef hamburger purchased from local markets and a one mL aliquot of *E. coli* O157:H7 suspension with known CFU were added. The media contained 4 mg/mL of sodium novobiocin (Sigma, St. Louis, MO) to prevent growth of non-*E. coli* bacteria. The hamburger suspension was shaken at 37C at 160 rpm for 6 h. Aliquots were removed at different time intervals and filtered through glass wool to remove large fat globules and meat particles. The filtered solutions were then treated with anti *E. coli* O157 IMB for Threshold System detection as described above.

Filter Membranes Replacement

Two types of nitrocellulose membrane sample sticks, with and without biotinylated BSA coating, can be purchased from Molecular Devices for the Threshold System apparatus. The "blank sticks" containing a 0.45 μm pore size nitrocellulose filter membrane without biotinylated BSA coating was used as sample sticks in present work. We also evaluated polycarbonate filter membrane (Osmonics Livermore, CA) as a possible replacement for the nitrocellulose filter membranes. After removing the nitrocellulose membrane filters, polycarbonate filters of 0.2 or 1.0 μm pore size were glued to the same location of the sticks using Elmers neoprene-based contact cement (Borden Inc, Columbus, OH). The modified sticks were then used in place of the "blank sticks" for Threshold System detection.

Other Materials

Analytical grades of sodium chloride, Tris, and urea were obtained from Sigma Chemical Company (St. Louis, MO). Beef hamburgers were purchased from local supermarkets.

RESULTS AND DISCUSSION

Application of IMB for LAPS Measurement by the Threshold System

In our previous report (Gehring *et al.* 1998), we demonstrated that between 10^4 to 10^5 CFU/mL of *E. coli* O157:H7 in solutions containing 150 mM NaCl and 10 mM tris, pH 7.5 (TBS), could be detected by the procedure recommended by the manufacturer of the Threshold System. In that procedure (Fig. 1, right side), the bacteria are first treated with a battery of antibodies, including biotin-labeled anti-*E. coli* O157:H7, fluorescein-labeled anti-*E. coli* O157:H7 and urease-labeled anti-fluorescein antibodies. For convenience, we refer to those steps as PHASE 1 for LAPS measurements. Streptavidin is then used to link the bacteria-antibody complexes to biotinylated bovine serum albumin that is coated on the surface of the nitrocellulose membranes. The complexes are then concentrated on the membranes by vacuum filtration. Since there is no provision for separating the targeted microbes before the filtration step, the filter membrane with pore size as 0.45 μm can be easily clogged by other particulate materials in complex samples. In our experience, the recommended procedure for the Threshold System becomes ineffective to detect *E. coli* O157:H7 spiked in pork carcass wash fluid. Thus, for complex food systems, alternative approaches are needed for applying the LAPS detection associated with the Threshold System.

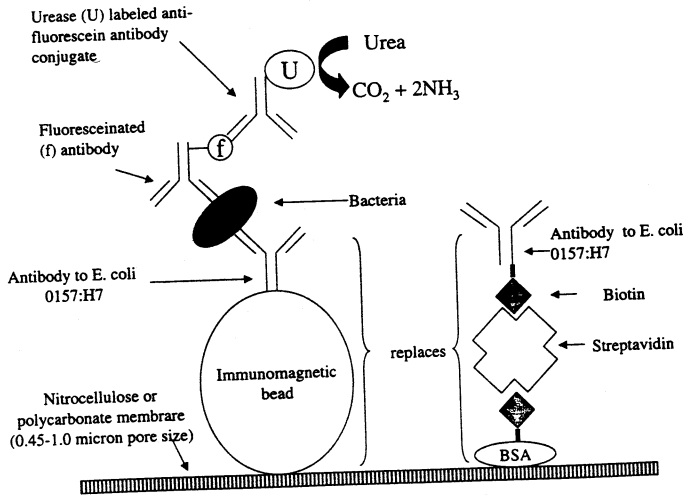

FIG. 1. SCHEMATIC REPRESENTATION OF APPLYING IMB FOR LAPS DETECTION
The details of the modifications were described in text. The standard procedure recommended by the instrument manufacturer is shown on the right side of the figure. The IMB replacement is illustrated on the left side of the figure.

One of the attractive methods is the application of IMB. IMB with specific antibodies have been applied to selectively concentrate targeted bacteria in different sample solutions (Gehring *et al.* 1996; Yu and Bruno 1996). We first used the specific IMB to concentrate *E. coli* O157:H7 in the sample solutions. The formed bacteria-IMB complexes could be effectively separated from other solution components by the use of a magnetic separator. The captured bacteria-IMB complexes could be considered as a replacement for the conjugates formed between biotinylated anti-*E. coli* O157:H7 and the bacteria in PHASE 1 of the above procedure. Because the applied spherical IMB had a diameter of 2.8 μm, the antibody-linked, bacteria-IMB complexes could then be retained by filter membranes having the proper pore sizes.

Furthermore, if the packing of the bacteria-IMB complexes to the membrane was tight enough, then LAPS detection of the bacteria would be possible without the use of the membrane containing biotin conjugated BSA. This IMB approach is also illustrated in Fig. 1 (left side). When this approach was applied to cultured *E. coli* O157:H7 of different concentrations in TBS, results shown in Fig. 2 were obtained. As shown, the modified approach showed provided a near linear response between the logarithm of the bacterial concentration (four to eight) and the logarithm of detected LAPS signals. The confident detection limit of this approach using the "blank sticks", is $\sim 10^5$ CFU of *E. coli* O157:H7 per mL of the initial bacterial suspension in TBS. Since the capture of the bacteria by the IMB is not 100% (Tu *et al.* 1998) the results also indicated the LAPS could detect the pH changes associated with $< 10^5$ CFU of the bacteria.

Choice of Filter Membrane

The results shown in Fig. 2 indicate that the IMB approach can replace the special filter membrane (nitrocellulose linked with biotinylated BSA) with a simple nitrocellulose membrane. Since membrane is used mainly to trap the IMB, the pore size of the membrane may not be critical for the experiment as long as the pore is less than the diameter of applied IMB (2.8 μm). We have replaced the nitrocellulose membrane with a polycarbonate membrane of various pore sizes to repeat the earlier experiments using cultured *E. coli* O157:H7 (Table 1). The results indicate that polycarbonate membrane with 0.2 or 1.0 μm pore size yielded a similar capture efficiency as that of nitrocellulose membrane with 0.45 μm pores. This result suggests that the use of the "blank sticks" provided by the instrument manufacturer is not necessary and other membrane filters may also be used for the developed IMB process.

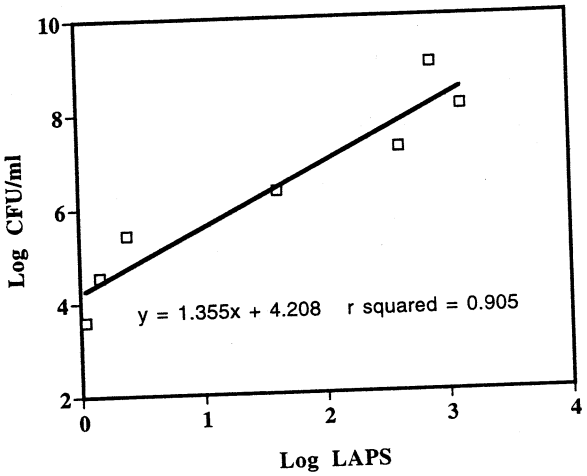

FIG. 2. LAPS DETECTION OF IMB CAPTURED *E. COLI* O157:H7
The bacteria isolated at the stationary phase were diluted to specified CFU per mL with TBS. To aliquots of 1 mL each of diluted samples, 10^6 IMB were added to capture the bacteria. The IMB captured bacteria were concentrated and further labeled with other conjugates as described in Materials and Methods before LAPS signals determined. The data represent average of triplicated experiments with errors as $\pm 10\%$.

TABLE 1.
CHOICE OF THE FILTER MEMBRANE FOR LAPS DETECTION[1]

Membrane Used	LAPS Signal ($\mu V\ s^{-1}$)
Nitrocellulose (0.45 μm)	160
Polycarbonate (0.20 μm)	133
Polycarbonate (1.0 μm)	145

[1] Pure culture of *E. coli* O157:H7 isolated at stationary phase was diluted to 10^6 CFU per mL with TBS. Aliquots of 100 μL of diluted bacterial suspensions were added to 900 μL of TBS containing 10^6 IMB and the mixtures were vortexed for 10 min. After concentrated by a magnetic separator, the IMB captured bacteria were treated with fluorescein-conjugated *anti-E. coli* serum and urease-conjugated anti-fluorescein serum and then were subjected to LAPS measurements as described in Materials and Methods. The LAPS signals of IMB-captured bacteria trapped on the membranes were detected. The data represent averages of triplicated experiments with errors as of $\pm$ 10%.

Application to Beef Hamburger System

Research from other laboratories (Tortorello and Gendel 1993; Tortorello and Steward 1994) indicate that the polyclonal anti-*E. coli* O157 antibodies used

to coat the utilized Dynal IMB, showed a minimal cross reactivity with other bacteria found in foods. In addition, the IMB retained their reactivity toward the *E. coli* in many complex food systems (Tu *et al.* 1998). The IMB application has also been incorporated with many biosensor detection methods to quantify the presence of specific pathogenic bacteria (Yu and Bruno 1996; Gehring *et al.* 1996). Since most of known *E. coli* O157:H7 outbreaks are linked to beef hamburger, we have chosen this meat system to test the developed IMB procedure for the Threshold System. We spiked beef hamburger patties with known but low dosages of the *E. coli*. The adulterated beef samples were then incubated in EC media for 6 h at 37C to enrich the bacterial population. After the enrichment, the *E. coli* cells were captured by the IMB. The bacteria-IMB complexes were then analyzed as described above. The results are shown in Fig. 3. After enrichment for 6 h at 37C, the procedure of using IMB to capture the bacteria and applying LAPS to monitor the level of the capture, was able to detect the presence of *E. coli* O157:H7 in the samples originally spiked with one CFU per gram of beef hamburger.

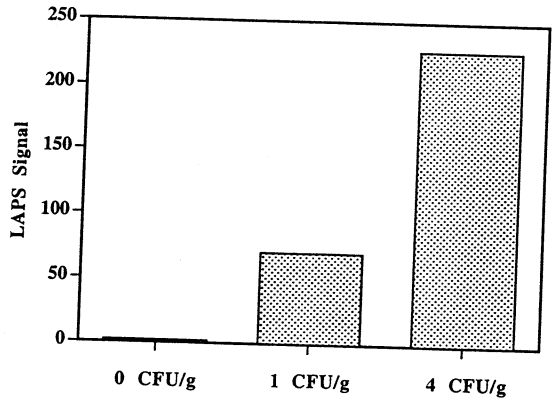

FIG. 3. LAPS DETECTION OF IMB-CAPTURED *E. COLI* O157:H7 FROM BEEF HAMBURGER

Beef hamburger patties (~ 25 g) were inoculated with indicated levels of the bacteria as described in text. After an enrichment for 6 h at 37C, the E. coli in 1 mL of glass-wool filtered media were captured by 10^6 IMB. The beads were then trapped on the nitrocellulose membrane for LAPS signal detection. The data shown represent averages of duplicated experiments with errors as ±10%.

The results shown in Fig. 3 indicate that a 6 h enrichment is sufficient for beef hamburger containing about one CFU of *E. coli* O157:H7 for every gram of the meat. Certainly, the length of the incubation period depends on the initial

concentration of the bacteria in the samples. Thus, we followed the growth kinetics of various "spiking" levels of the bacteria in beef hamburger patties (Fig. 4). The results indicate that the enrichment time-period for generating sufficient concentrations of the bacteria to yield positive LAPS responses varied with the spiking level of the *E. coli* in the hamburger. The data show that with one CFU per gram of beef hamburger, six hours enrichment is necessary. However, with 40 CFU per gram of the same hamburger, enrichment period may be shortened to four hours.

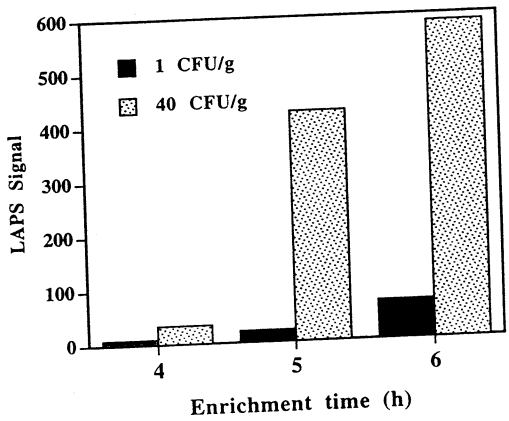

FIG. 4. LAPS SIGNALS OF IMB-CAPTURED *E. COLI* O157:H7 FROM BEEF HAMBURGER WITH DIFFERENT ENRICHMENT PERIOD

Beef hamburger patties were spiked with indicated levels of *E. coli* O157:H7. The bacteria samples in the enrichment media were withdrawn at indicated time intervals and they were subjected to IMB capture process. The LAPS signals represent the averages of duplicated experiments with errors as of ±10%.

CONCLUSION

We have shown that the described method of using IMB to capture the targeted bacteria for LAPS detection has a similar sensitivity as that found for using the complete commercial assay kit. This modification allows us to omit the use of biotin-streptavidin reactions to conjugate the bacteria and the filter membranes. In addition, IMB may be used directly in complex foods, such as beef hamburger system to capture the targeted pathogenic bacteria. Subsequent separation of IMB-captured bacteria from other components in the sample suspensions may use filter of larger pore size and thus, increases the speed of analysis. With the use of IMB, the LAPS measurement of the Threshold instrument may be adapted for bacterial detection in food systems.

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